INCREASING POTENCIES OF ENZYMES PRODUCED BY ASPERGILLUS NIGER[1]

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Work at the Northern Regional Research Laboratory has established the usefulness of *Aspergillus niger* in the production of amylases (Le Mense et al. (3)). In adapting these methods to production of amylases on potato substratum, it became evident that certain modifications were required. Calcium carbonate in the potato medium significantly decreased enzyme potencies, as was found also by Tsuchiya et al. (6) of the Northern Regional Research Laboratory. *A. niger* grew vigorously on potato substratum, but filtrates of fermentations showing extremely abundant mycelium after short incubation periods had low enzyme potencies. Work was therefore initiated to obviate this condition on the theories that: (a) Young active mycelium liberates minimum quantities of enzymes into solution, and conversely, dead mycelium liberates maximum quantities of enzymes; and (b) comminution of the mycelium increases enzyme potencies by releasing cell contents.

Aspergillus niger NRRL No. 330 was grown on 6 per cent whole potato flour with an inoculum of three million spores per ml. of medium and incubated at 30° C. on a reciprocal shaker. Enzyme potencies of all cultures whether treated or untreated were determined on the supernatant liquid obtained after centrifuging. Starch conversion was determined by the ferricyanide method reported by Erb, Wisthoft and Jacobs (2) except that sulfuric acid was omitted from the recommended buffer solution. Maltase was determined by a copper reduction method, essentially that of Somogyi (5), supplied by Dr. Henry M. Tsuchiya of the Northern Regional Research Laboratory, which measures milligrams of maltose hydrolyzed per ml. of culture per hour.

Many of the methods used for preparing enzymes from yeasts and bacteria were tried. These methods are reviewed by Umbreit et al. (7), Werkman and Wood (8), and Bernhauer and Knobloch (1). Except for the homogenizer described by Potter and Elvehjem (4), none of these methods gave satisfactory increases in enzyme potencies. Our objective of obtaining a method which could be adapted to commercial use was of paramount importance in evaluating the usefulness of the above methods. Several mechanical methods of extracting amylases from *A. niger* mycelium are given in table I. A modified Logeman hand homogenizer gave

TABLE I

EFFICIENCY OF DIFFERENT MECHANICAL METHODS FOR EXTRACTING AMYLASES FROM *Aspergillus niger* MYCELIUM

Sample	Treatment*	Starch conversion		Maltase	
		%	% increase	†	% increase
1	None	51.16	—	13.4	—
	A	53.34	4.3	12.5	0
	B	57.00	11.4	32.6	143.1
2	None	22.50	—	5.3	—
	B	30.83	37.0	17.3	226.4
	C	27.58	22.6	7.3	37.1
	D	28.75	27.8	8.9	67.4

* A = Processed for 20 minutes at high speed in a water-cooled Waring Blendor.
B = Processed five times in a *modified* Logeman hand homogenizer.
C = Processed for 20 minutes in a Charlotte Colloid Mill, Model A, set at 0.001 inch.
D = Processed five times in the Logeman hand homogenizer.
† Milligrams of maltose hydrolyzed per ml. of culture per hour.

satisfactory increases in enzyme potencies and was selected for comminution of the mycelium over the other treatments investigated. This Logeman hand homogenizer was modified by grinding both pressure plates flat, increasing tension on the spring, placing rings on the piston and reinforcing the handle. It was estimated that this modified homogenizer comminuted the mycelium so that about 10 per cent was less than 10 microns long, whereas the other treatments mentioned in table I seldom produced hyphae less than 50 microns long. This action is coincident with the increased liberation of starch-converting enzymes and maltase over the other treatments of table I.

As shown in table II, comminuting different fermented potato media in the modified homogenizer consistently produced increased amylase potencies. Lack of pH control decreased enzyme potencies, which has been shown previously by Tsuchiya et al. (6). The pH of Samples 1 to 5 was not under adequate control. Good pH control was maintained throughout the incubation period for Samples 6 to 9. Enzyme potencies of samples held for longer incubation periods showed that starch-converting enzymes were liberated in good supply without comminution but maltase was still significantly increased by comminution.

TABLE II

INCREASE IN AMYLASE POTENCIES DUE TO COMMINUTION OF *Aspergillus niger* MYCELIUM

Sample	Incubation time, hours	pH >4.40 during incubation	Starch conversion, %			Maltase		
			Not com.*	Com.*	Increase	Not com.†	Com.†	% increase
1	17	No	—	—	—	0.4	6.6	1780.0
2	18	No	10.7	15.2	42.2	3.7	12.3	231.1
3	18	No	7.7	11.2	45.6	4.1	19.3	376.5
4	18	No	18.8	24.5	30.7	9.4	21.6	130.8
5	18	No	13.3	16.7	25.8	3.5	7.9	125.6
6	21	Yes	13.1	22.3	70.7	8.6	19.4	126.9
7	31	Yes	—	—	—	14.2	30.4	113.8
8	32	Yes	51.2	57.0	11.4	13.4	32.6	143.1
9	41	Yes	56.0	60.8	8.6	14.8	36.4	146.1

* Com. = comminuted, that is processed five times in the modified homogenizer.

† Milligrams of maltose hydrolyzed per ml. of culture per hour.

Killing the fungus with fungicides did not give a significant increase in maltase immediately but produced a significant increase in soluble maltase on storage (TABLE III). Storing the control (fermented medium, fungus not killed) in a refrigerator for extended periods of time did not produce an appreciable increase in maltase. Ammonium bifluoride was the best fungicide tried for killing the fungus. Merthiolate, as well as several other fungicides used, had inhibitory action on the maltase.

As shown in table III, comminution of the dead mycelium in a fermented medium produced significant increases in enzyme potency, even after storage of the dead mycelium for as long as 18 days. These studies indicate that the maltase enzyme system has

limited permeability to both the living and dead cell membranes, or that (a) maltase is merely occluded in the protoplasm and additional quantities are set free on release of protoplasm from the cell, or (b) maltase is bound to materials not permeable to the cell membrane, so that release of the cell protoplasm permits activity even though the maltase is bound to a constituent(s) of the protoplasm.

TABLE III

EFFECTS OF CHEMICAL AND MECHANICAL TREATMENTS OF *A. niger* MYCELIUM ON LIBERATION OF SOLUBLE MALTASE

Sample	Storage	Fungicide	Maltase values*		
			Not com.	Com.	% increase due to com.
1	A. 18 hrs. at 30° C.	None	18.1	—	—
	B. 18 hrs. at 30° C.	0.1% merthiolate	17.3	—	—
	AB. % increase due to fungicide		−4.4	—	—
	C. 14 days at 4° C.	None	18.5	22.5	21.9
	D. 14 days at 30° C.	0.1% merthiolate	23.9	27.1	13.5
	CD. % increase due to fungicide at 14 days		29.4	20.5	—
2	A. 72 hrs. at 30° C.	None	5.8	8.9	54.5
	B. 72 hrs. at 30° C.	0.1% ammonium bifluoride	7.3	17.3	138.0
	AB. % increase due to fungicide		26.4	94.7	—
	C. 18 days at 30° C.	0.1% ammonium bifluoride	10.3	20.4	98.8
	AC. % increase due to fungicide†		78.5	129.7	—

* Milligrams of maltose hydrolyzed per ml. of culture per hour.
† Based on results obtained in 2A.

These results direct attention to the important effect of pretreatment of the fermented medium on enzyme potencies. Methods which employ culture filtrates for determination of enzyme potency can supply misleading information on the potential quantities of enzymes in a fermented medium. By using a culture filtrate of a fermentation, it is possible to obtain results which indicate that a poor fermentation with a high proportion of dead mycelium has a higher concentration of enzymes than a vigorous fermentation with

a low proportion of dead mycelium. Actually, in absolute terms, the vigorous fermentation might have the higher enzyme content because of the greater quantity of mycelium. There is need of a simple, efficient means of releasing the entire cell contents of fungus mycelium without inactivation of the desired product. With such a method available, the efficiency of proposed industrial operations for the release of the enzyme could be more accurately evaluated.

It is believed that the mechanical and chemical treatments reported here for increasing the concentration of soluble enzymes from young, active mycelium release only a fractional quantity of the enzymes in the mycelium. Increased efficiency of comminution and more suitable fungicides are expected to give increased enzyme potencies. Proper comminution of the mycelium would provide both a killing action on the mycelium and slice the cell, liberating the cell contents.

Other methods of treating *A. niger* mycelium to increase enzyme potencies, such as plasmolysis, enzymolysis, and increasing cell membrane permeability, are being studied.

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